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Quantitative Determination of Ecteinascidin 743 in Human Plasma by Miniaturized High-performance Liquid Chromatography Coupled with Electrospray Ionization Tandem Mass Spectrometry

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A method was developed for the bio-analysis of Ecteinascidin 743 (ET-743) using miniaturized liquid chromatography (LC) coupled to an electrospray ionization sample inlet (TurbolonSpray) and two quadrupole mass analyzers (LC/ESI-MS/MS). Solid-phase extraction was used as a sample pretreatment procedure. Ecteinascidin 743 is a very potent anticancer compound and is administered in μg m^{-2} dosages, which demands special requirements in terms of sensitivity for the analytical method supporting clinical pharmacokinetic studies. Using conventional LC/UV, a lower limit of quantitation (LLQ) of 1 ng ml^{-1} plasma was reached using a 500 μl sample volume, but LC/ESI-MS/MS permitted an LLQ of 10 pg ml^{-1}. The latter method was found to be accurate and precise, and provided a broad linear concentration range of 0.010–2.50 ng ml^{-1}. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: Ecteinascidin 743; bio-analysis; human plasma; electrospray ionization; tandem mass spectrometry

INTRODUCTION

The development of potent anticancer drugs in conjunction with their administration in the clinical setting by prolonged infusion schedules has made the determination of these drugs in biological fluids by conventional liquid chromatography (LC) extremely difficult. The recent development of extremely potent anticancer compounds has brought forth a need for analytical equipment with capabilities down to at least the pg ml^{-1} level. The introduction of capillary/miniaturized LC columns has provided an opportunity to combine these types of chromatography with efficient non-destructive sample inlets into the mass spectrometer to achieve extremely sensitive and specific drug detection.

Ecteinascidin-743 (ET-743, Fig. 1) is a novel marine-derived anticancer chemical entity isolated from the ascidian *Ecteinascidia turbinata*, a Caribbean tuni-

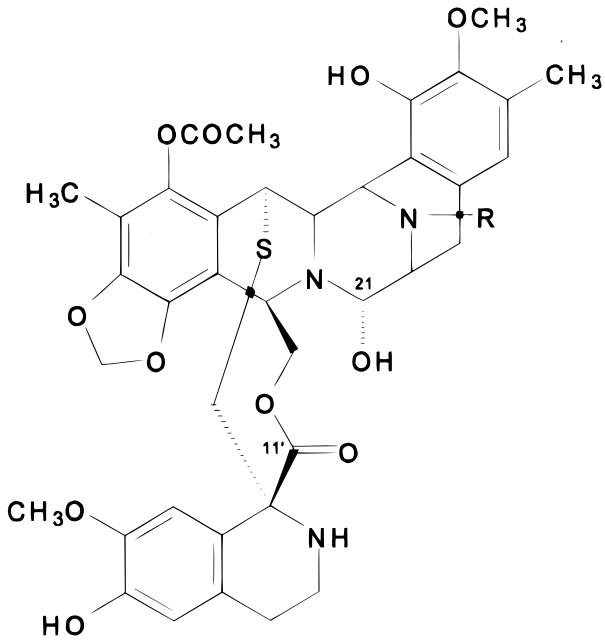

Figure 1. Structures of ET-743 (R = CH_3) and ET-729 (R = H).

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cate.[1,2] The drug has demonstrated significant activity in nude mice bearing human tumors including breast, non-small cell lung, ovarian, melanoma and renal cancers. ET-743 exerts its antitumor activity by interacting with minor groove DNA guanine-rich sequences and modifying the cell microtubule network.[3] The precise mechanism of action is not known and is still under investigation.[4–6] The drug is now entering phase II clinical trials in Europe and the USA.

For the quantification of ET-743 in human plasma, we have reported a conventional LC/UV assay, utilizing solid-phase extraction (SPE) on cyano columns as a sample pretreatment procedure.[7] The assay quantitates ET-743 concentrations of 1.0–50.0 ng ml^{-1} using a 500 μl sample volume. This assay has been used to support pharmacokinetic research in a phase I study, where ET-743 was administered in 1 h infusions. At the starting dose (50 μg m^{-2}) in this study only maximum concentrations of ET-743 at the end of infusion could be quantified in the plasma of patients. When 24 h infusions every 21 days and five times daily 1 h infusion schedules were tested in the clinic at starting doses of 50 and 6 μg m^{-2}, respectively, no drug could be detected in the plasma of the treated patients. Therefore, an analytical method with higher sensitivity was developed to monitor complete pharmacokinetic profiles at these low dosages. The use of miniaturized LC combined with electrospray ionization (TurbolonSpray) and mass spectrometry (MS) was essential to overcome these sensitivity problems. In this study, we have developed and validated a miniaturized LC electrospray ionization (ESI)-MS/MS method for the quantification of ET-743 in human plasma. The applicability in the context of a phase I clinical program is demonstrated.

EXPERIMENTAL

Chemicals

ET-743 (lot IMS 196) reference and ET-729 (lot FC-44/729, internal standard for the LC/ESI-MS/MS method, Fig. 1) were obtained from Pharma Mar (Madrid, Spain). Acetonitrile (LC gradient grade) was obtained from Biosolve (Amsterdam, The Netherlands) and methanol (ChromAR) from Promochem (Wesel, Germany). Ammonium acetate, glacial acetic acid, 37% hydrochloric acid, formic acid, disodium hydrogenphosphate dihydrate and sodium dihydrogenphosphate dihydrate (all of analytical grade) were purchased from Merck (Darmstadt, Germany). Doubly distilled water was used throughout. Drug-free human plasma originated from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Instrumentation

The LC system consisted of a Perkin-Elmer (PE) (Norwalk, CT, USA) Model 200 series pump and an ISS 200 autosampler. A methanol-water (75:25, v/v) mixture containing 5 mM ammonium acetate and 4‰ (v/v) formic acid was used as the mobile phase. At a flow-rate of 200 μl min^{-1}, the mobile phase was pumped through a Zorbax Rx-C18 column (15 × 2.1 mm i.d., particle size 5 μm (Rockland Technologies, Newport, DE, USA) protected with an in-line filter (0.2 μm) (Upchurch Scientific, Oak Harbor, WA, USA). The column outlet was connected directly to the TurbolonSpray sample inlet (Sciex, Thornhill, ON, Canada) without splitting. Ions were created at atmospheric pressure and were transferred to an API 365 triple-quadrupole mass spectrometer (Sciex). Major parameters used in this study are listed in Table 1.

Preparation of stock solutions, working solutions and plasma standards

Two ET-743 stock solutions were prepared in methanol at concentrations of 2.0 mg ml^{-1}. One solution was prepared to spike the plasma calibration standards and the other was prepared independently for the quality control samples. Both stock solutions were diluted further with methanol to obtain working solutions with concentrations of 100 and 10 μg ml^{-1}, respectively.

ET-729 stock solution was prepared in methanol at a concentration of 1.0 mg ml^{-1}. The stock solution was diluted further with methanol to obtain a working solution of 2.5 μg ml^{-1}. All solutions were stored at approximately −30 °C and were stable for at least 2 months. A reconstitution solvent was prepared as follows: the ET-729 working solution was diluted 1000-fold in

Table 1 ESI-MS/MS settings

Parameter	Setting
Electrospray voltage (positive ion mode)	+1500 V
Orifice voltage	+46 V
Ring voltage	+280 V
Quad O	−10.5 V
Inter quad 1	−14.5 V
Stubbies	−15.5 V
Rod offset 1	−11.5 V
Inter quad 2	−28 V
Rod offset 2	−51 V
Inter quad 3	−76 V
Rod offset 3	−56 V
Deflector	−380 V
Channel electron multiplier	1900 V
Dwell time	800 ms
Nebulizer gas (compressed air) flow-rate	1.8 l min^{-1}
Curtain gas (N_2 5.0) flow-rate	1.2 l min^{-1}
Turbo gas (N_2 5.0) flow-rate	7 l min^{-1}
Temperature	500 °C
Collision activated dissociation gas (N_2 5.0) flow-rate	0.3 l min^{-1}
ET-743	
Quad 1 selected mass	744.4 u
Quad 2 selected mass	495.0 u
ET-729	
Quad 1 selected mass	730.6 u
Quad 2 selected mass	478.8 u

methanol–water (50:50, v/v) containing 5 mM ammonium acetate to achieve an ET-729 concentration of 2.5 ng ml^{-1}. This solvent was used to redissolve the dry extracts after SPE. The solution was stored at approximately −30 °C and was stable for at least 24 h.

A plasma standard of 100 ng ml^{-1} was prepared by adding 50 µl of the ET-743 working solution (10 µg ml^{-1}) to 5.0 ml of control human plasma. This standard (100 ng ml^{-1}) was further diluted in control human plasma to achieve analyte concentrations of 0.01, 0.05, 0.25, 0.80, 1.80 and 2.50 ng ml^{-1}. Plasma calibration standards were prepared freshly for each analytical run.

Sample processing

Before extraction, the SPE columns (non-end-capped cyano columns, 100 mg) (Varian, Harbour City, CA, USA) were first activated and washed. This was performed successively with 1 ml of 0.1 M hydrochloric acid in methanol, 2 ml of methanol and 2 ml of 0.01 M ammonium acetate (pH 5.0). A 1000 µl sample volume (plasma–0.2 M ammonium acetate (pH 5.0) (1:1, v/v)) was loaded on to the columns with a dispense flow-rate of ~1 ml min^{-1}. After successive washings with 2 ml of 0.01 M ammonium acetate (pH 5.0) and 2 ml of acetonitrile, elution of ET-743 was performed with 1 ml of 0.1 M hydrochloric acid in methanol. The elution solvent was evaporated to dryness under a nitrogen stream at ~40 °C. The dry residues were dissolved in 200 µl of the reconstruction solvent by mixing the tubes for 1 min. Before injecting a volume of 20 µl on to the LC column, the sample was filtered over a PTFE filter (Alltech, Deerfield, IL, USA).

Validation procedures

Linearity The plasma calibration standards (see above) were analyzed in duplicate in three separate analytical runs. To account for the fact that the variance over the range of calibration is not constant, the data obtained (linear regression of the ratio of the areas of the analyte and internal standard peaks versus the concentration) were weighted by the reciprocal of the squared concentration to increase the accuracy at low concentrations. The F test for lack of fit ($\alpha = 0.05$) was used to evaluate the linearity of the calibration curves.[8]

Accuracy and precision. Quality control samples at four levels, distributed over the concentration range, were prepared in control human plasma by dilution of an independently prepared plasma standard containing 100 ng ml^{-1} (see above). Each quality control sample was processed and analyzed six times in three different runs with plasma calibration standards to determine the ET-743 concentration. The accuracy was calculated at each test concentration as the ratio of the measured concentration and the nominal concentration multiplied by 100%. The assay precisions were obtained by one-way analysis of variance (ANOVA) for each test concentration using the run day as the classification variable (software package Statistical Product and Service Solutions, version 6.1 for Windows, SPSS, Chicago, IL, USA). The following equations were used to calculate the precisions:

$$\text{within-run precision} = \frac{\sqrt{MS_{WG}}}{GM} \times 100\%$$

$$\text{between-run precision} = \frac{\sqrt{\frac{MS_{BG} - MS_{WG}}{n}}}{GM} \times 100\%$$

where, MS_{WG} is the mean square of the within-groups/runs, MS_{BG} is the mean square of the between-groups/runs, GM is the grand mean of the measured quality control concentration and n is the number of determinations per group/run. The acceptance criteria are not more than 15% for accuracy and precision. At the lower limit of quantitation, 20% is acceptable for both accuracy and precision.[9]

Selectivity and specificity. To investigate whether endogeneous compounds interfered with the assay, six batches of control human plasma were processed and analyzed. Co-medicated drugs were added to control human plasma in therapeutic concentrations and were then processed and assayed according to the described method to investigate co-elution of these drugs with the analyte and/or internal standard. The following drugs were tested: acetaminophen (20 µg ml^{-1}), morphine (50 ng ml^{-1}), dexamethason (100 ng ml^{-1}), ondansetron (40 ng ml^{-1}), domperidone (500 ng ml^{-1}), metoclopramide (50 ng ml^{-1}) and omeprazole (200 ng ml^{-1}).

Recovery. To determine the overall extraction recoveries, the slopes of the processed calibration curves were compared with the slopes of the standard curves prepared in reconstitution solvent ($n = 3$).

Stability. The stability of ET-743 in methanol was investigated at a concentration of 10 µg ml^{-1} when stored at approximately −30 °C for 2 months. In plasma, the stability of the drug was studied at concentrations of 20 and 40 ng ml^{-1} for 1.5 years at approximately −30 °C, and at ambient temperatures at a concentration of 10 ng ml^{-1} for 24 h. The processed sample stability of ET-743 in plasma extracts after dissolution in reconstruction solvent was studied at a concentration of 0.8 ng ml^{-1} for 12 h.

Robustness. Several Zorbax Rx-C18 columns (15 × 2.1 mm i.d., particle size 5 µm, serial numbers NC1530, NC1850, NC1852 and NC1857) were tested during the validation phase and application phase of the method.

Pharmacokinetic case studies

Three patients with solid tumors were treated with ET-743 in a phase I clinical study at dose levels of 100, 600 and 1200 µg m^{-2} given as a 24 h infusion. Whole blood samples were taken prior to the start of the infusion and at 2, 6 and 23.5 h after the start of the infusion. Post-infusion samples were taken at 5, 10, 15, 30 and 60 min and 2, 4, 6, 9, 12 and 24 h. Blood samples (8 ml) were collected in heparinized tubes and immediately

centrifuged (15 min at 4000 *g*). The plasma layer was then removed and stored at approximately −30 °C until analysis.

Pharmocokinetic parameters were obtained using a model-independent approach. For each patient, the maximum drug concentration (C_{max}) and the time to maximum drug concentration (t_{max}) were generally directly from the experimental data. The total area under the curve ($AUC_{0\rightarrow\infty}$) was calculated using the trapezoidal rule with extrapolation to infinity using the terminal rate constant k (C_{last}/k, where C_{last} is the last measured concentration). The elimination half-life ($t_{1/2}$) was calculated as $0.693/k$ and the total plasma clearance (Cl_{tot}) as the dose divided by the $AUC_{0\rightarrow\infty}$. The volume of distribution (V) was determined using the equation $V = Cl_{tot}/k$.

RESULTS

Optimization of the LC/ESI-MS/MS system

The column outlet was connected directly to the TurbolonSpray sample inlet without splitting. A mass spectrum of ET-743 (molecular mass 762 Da) transmitted by the first mass analyzer shows ions at *m/z* 744 and 776 (Fig. 2). From this spectrum, it can be concluded that collisionally induced dissociation (upfront dissociation) of the analyte ions takes place; a water molecule is lost from the parent molecule. This effect could not be prevented by lowering the orifice and/or pre-quadrupole voltage. ET-743 derived its name from this dissociated parent molecular ion at *m/z* 744. The ion at *m/z* 776 in the mass spectrum may be explained by the formation of methanol cluster ions in the ion source or by the presence of a methoxy derivative in the eluent. The carbinolamine moiety is very reactive and in the presence of methanol a substitution of a methoxy group at C-21

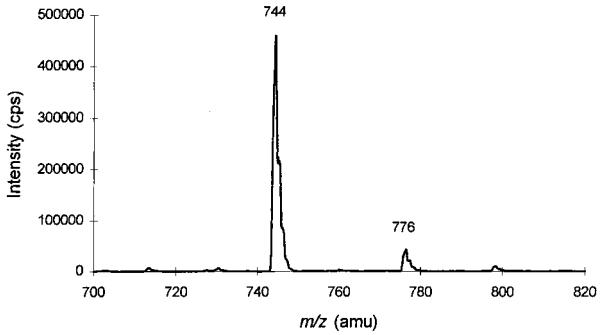

Figure 2. An electrospray mass spectrum of ET-743 transmitted by the first mass analyzer at *m/z* 700–820 with a step-size of 0.5 u acquired with a dwell time of 10 ms.

has been proposed (Fig. 1).[2,10] After selecting *m/z* 744 ions by the first mass analyzer, fragment ions at *m/z* 495 were detected. This fragment ($C_{27}H_{31}N_2O_7$) was formed in the collision cell after cleavage of the sulfur bond and ester binding at C-11′.[2]

The effects of methanol content, ammonium acetate concentration and percentage of acetic acid in the mobile phase on the ESI response (*m/z* 744–495 transition) were investigated and results are plotted in Fig. 3. A higher proportion of methanol increases the signal intensity. Better spray performance appears to be due to the lower surface tension of the organic modifier. An optimum was reached at ~80% (v/v) of methanol. The addition of ammonium acetate to the mobile phase decreases the response intensity dramatically. Ion competition in the droplet surface may be the major factor controlling the ionization process. A concentration of 5 mM ammonium acetate was chosen for the assay, because some buffer capacity in the mobile phase was needed to achieve a reproducible separation between ET-743 and the internal standard. Electrospray sensitivities were also greatly reduced by increasing the amount of acetic acid in the mobile phase. The use of formic acid, however, led to a significant enhancement

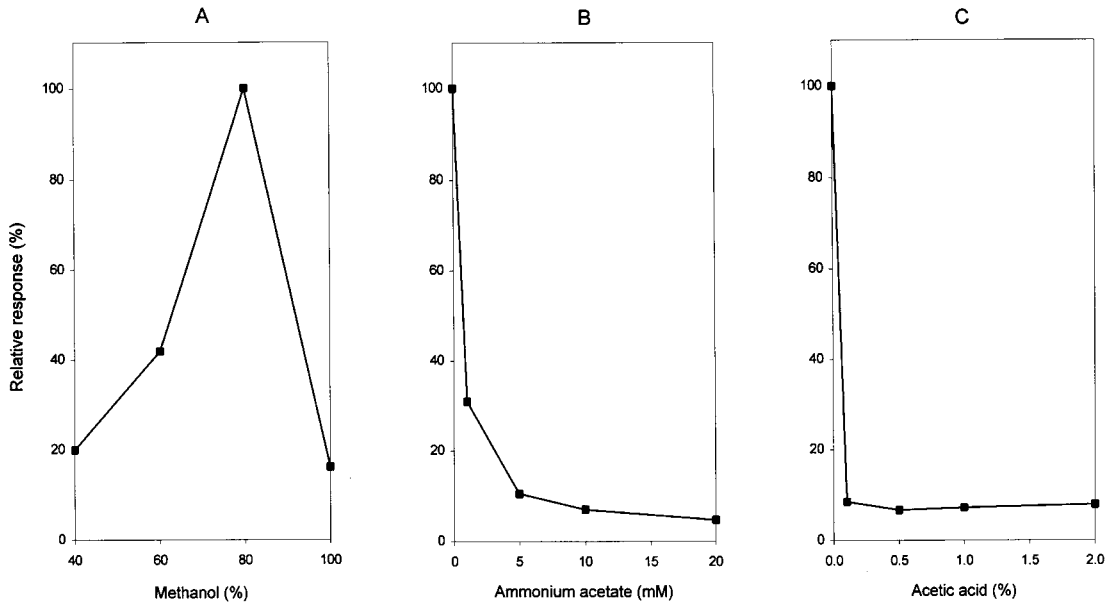

Figure 3. Effect of (A), methanol content, (B) ammonium acetate concentration and (C) percentage of acetic acid in the mobile phase on the relative ion response in ESI-MS/MS.

of the sensitvity. A concentration of 4‰ (*v*/*v*) formic acid gave the best response. Fig. 4 presents typical selected ion chromatograms for the mass spectrometric assay.

Validation of the assay

The assay was linear over the tested concentration range in human plasma, as determined by the *F* test for lack of fit ($\alpha = 0.05$). For each calibration curve the calibration concentrations were back-calculated from the ratio of the peak areas of ET-743 and the internal standard (see Table 2). The deviation of the nominal concentration for all concentrations was $\leqslant 5\%$.

Assay performance data are presented in Table 3. At the lower limit of quantification (0.010 ng ml^{-1}) the accuracies and precisions were less than the required 20% level[9]. The accuracies and precisions for the other tested concentrations were all within $\pm 10\%$.

Chromatograms of six batches of control human plasma samples contained no endogenous peaks co-eluting with ET-743 and/or ET-729. No chromatographic interferences were found from the tested concomitant drugs. The mean overall extraction recovery of ET-743 from human control plasma was $98.8 \pm 10.5\%$ ($n = 3$).

In Table 4 the stability data for ET-743 are summarized. ET-743 was found to be stable in human plasma for at least 1.5 years, when stored at $-30\,^{\circ}C$.

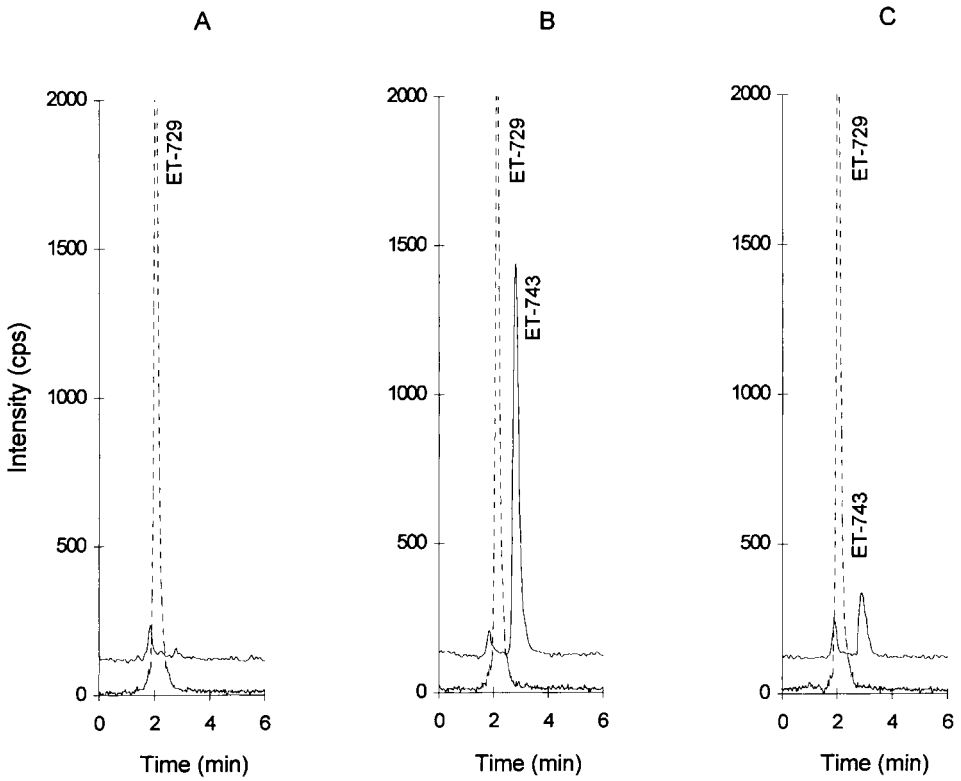

Figure 4. Selected ion chromatograms typical for the LC/ESI-MS/MS determination of ET-743 in human plasma: blank plasma (A) and plasma samples collected at the end (B, 0.1088 ng ml^{-1}) and 48 h after cessation of a 24 h infusion at a dose level of 100 μg m^{-2} (C, 0.0183 ng ml^{-1}). The solid line represents the transition of *m*/*z* 744.4 to 495.0 (ET-743) and the dotted line *m*/*z* 730.6 to 478.8 (internal standard ET-729).

Table 2 Calibration concentrations ($n = 2$) back-calculated from the ratio of the peak areas of ET-743 and ET-729

	Concentration (ng ml^{-1})					
	0.01	0.05	0.25	0.80	1.80	2.50
1	0.0101	0.0463	0.259	0.865	1.960	2.137
2	0.0102	0.0467	0.232	0.759	1.834	2.886
3	0.0100	0.0494	0.242	0.789	1.789	2.647
Mean	0.0101	0.0475	0.244	0.804	1.861	2.557
RSD (%)[a]	1.0	3.6	5.6	6.8	4.8	14.9
Deviation (%)	1.0	−5.0	−2.4	0.5	3.4	2.3

[a] RSD = relative standard deviation. Deviation = deviation of the nominal concentration.

Table 3 Assay performance for the determination of ET-743 in human plasma

Nominal concentration (ng ml)$^{-1}$	Measured concentration (ng ml)$^{-1}$	Accuracy (%)	Within-day precision (%)	Between-day precision (%)	No. of replicates
0.010	0.0102	102	9.3	5.7	18
0.025	0.0252	101	8.2	5.3	18
1.00	1.0309	103	5.9	4.4	18
2.50	2.4125	97	5.1	4.2	18

Table 4. Stability data for ET-743

Storage conditions	Initial concentration (ng ml^{-1})	Recovery (%)	RSD (%)	No. of replicates	Assay system
Methanol at −30 °C for 2 months	10000	99	2.3	2	LC/UV[7]
Plasma at −30 °C for 1.5 years	40.0	94	2.4	3	LC/UV[7]
	20.0	93	2.4	4	
Plasma at ambient temperatures for 5 h	10.0	99	1.0	2	LC/UV[7]
Plasma at ambient temperatures for 24 h		91	4.6	2	
Reconstitution solvent after plasma extraction at ambient temperatures for 12 h	0.8	102	4.6	2	LC/ESI-MS/MS (this work)

After 24 h at ambient temperatures the concentration of the drug was 91 ± 4.6% of the initial concentration. It is recommended that the sample preparation procedure be performed within 5 h (Table 4). In reconstitution solvent ET-743 was found to be stable for at least 12 h. This allows the use of an autosampler injection device for the assay.

Several analytical column batches were used during the validation phase and application phase of the method. For all columns tested identical separation performances were observed for the analyte and the internal standard.

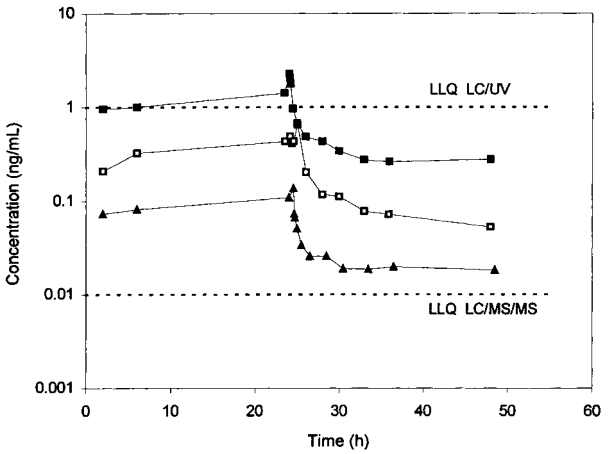

Figure 5. Plasma concentration *vs* time profiles of ET-743 in patients treated with (▲) 100, (□) 600 and (■) and 1200 μg m^{-2} administered as a 24 h infusion. The dotted lines represent the lower limit of quantitation (LLQ) of the conventional LC/UV method[7] and the described LC/MSMS assay (1.0 and 0.01 ng ml^{-1}, respectively).

Table 5 Pharmacokinetics of ET-743

	Patient No.		
Pharmacokinetic parameter	4	13	20
Dose (μg m^{-2})	100	600	1200
Total dose (μg)	160	900	2256
t_{max} (h)	24.6	25.1	24.1
C_{max} ng ml^{-1}	0.137	0.656	2.287
$AUC_{0\rightarrow\infty}$ h μg l^{-1}	4.459	12.29	52.43
Half-life, $t_{1/2}$ (h)	67.1	17.5	41.6
Clearance, CL l h^{-1} m^{-2}	22.4	48.8	22.9
Distribution volume, l m^{-2}	2171	1235	1375

Pharmacokinetic case studies

To show the applicability of the method, the plasma concentration *vs* time profiles of ET-743 in three patients treated with dose levels of 100, 600 and 1200 μg m^{-2} in 24 h infusions are presented in Figure 5. The relevant pharmacokinetic parameters of ET-743 are given in Table 5. The maximum concentrations (C_{max}) were found at the end of the ET-743 administrations. Post-infusion, the drug concentration declines rapidly. In the second phase, the elimination is prolonged: for one patient a half-life of 45 h was calculated.

ET-743 might undergo *N*-dealkylation as a metabolic pathway to form ET-729. This compound was used as internal standard for the assay, as stable isotopes of ET-743 were not available. Several plasma samples from each patient were analyzed without addition of the internal standard to check for the possible presence of ET-729 in the samples. No indications of ET-729 metabolism were found.

DISCUSSION

ET-743 is a marine-derived anticancer entity that has been identified, isolated, purified and developed for potential use as an anticancer drug. The potency of the drug presents an analytical problem, especially when

the drug is administered at low μg m^{-2} doses in prolonged infusion schedules. Plasma levels were far below the lower limit of quantification of the conventional LC/UV method (Fig. 5). To overcome this problem, an analytical system which combines miniaturized LC with ESI-MS/MS was designed. This system was found to provide detection capabilities down to 10 pg ml^{-1} with excellent accuracy and precision. The sensitivity was improved by a factor 100 compared with the conventional LC/UV method. In addition to enhancement of sensitivity, specificity of detection is also improved. The low solvent flow-rate of 200 μl min^{-1} typically used with miniaturized LC is ideally suited for ESI. The 2.1 mm i.d. columns provide high theoretical plate numbers and permit short run times of ~5 min. ET-729 was added to the plasma extract after the sample pretreatment procedure.[7] Although losses during the extraction were not compensated, the validation outcomes showed that the assay was accurate and precise. The semi-internal standard ET-729 was used to correct for stability in injection volumes and ionization conditions.

In conclusion, an accurate, reproducible and specific LC/ESI-MS/MS assay, utilizing SPE on cyano columns as a sample pretreatment procedure, was developed for the quantification of ET-743 in human plasma. The assay quantitates ET-743 concentrations of 0.01–2.5 ng ml^{-1} using a 500 μl sample volume. This assay is now being used to support pharmacokinetic research in phase I studies. The present work demonstrates that LC/ESI-MS/MS is a useful analytical approach for the quantitation of pharmaceutical compounds in biological matrices which need to be determined at extremely low levels.

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